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Isolation and Characterization of Bacteria Capable of Tolerating the Extreme Conditions of Clean Room Environments[▽]

Myron T. La Duc, Anne Dekas, Shariff Osman, Christine Moissl,† David Newcombe, and Kasthuri Venkateswaran*

Biotechnology and Planetary Protection Group, Jet Propulsion Laboratory, California Institute of Technology, Pasadena, California

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In assessing the bacterial populations present in spacecraft assembly, spacecraft test, and launch preparation facilities, extremophilic bacteria (requiring severe conditions for growth) and extremotolerant bacteria (tolerant to extreme conditions) were isolated. Several cultivation approaches were employed to select for and identify bacteria that not only survive the nutrient-limiting conditions of clean room environments but can also withstand even more inhospitable environmental stresses. Due to their proximity to spacefaring objects, these bacteria pose a considerable risk for forward contamination of extraterrestrial sites. Samples collected from four geographically distinct National Aeronautics and Space Administration clean rooms were challenged with UV-C irradiation, 5% hydrogen peroxide, heat shock, pH extremes (pH 3.0 and 11.0), temperature extremes (4°C to 65°C), and hypersalinity (25% NaCl) prior to and/or during cultivation as a means of selecting for extremotolerant bacteria. Culture-independent approaches were employed to measure viable microbial (ATP-based) and total bacterial (quantitative PCR-based) burdens. Intracellular ATP concentrations suggested a viable microbial presence ranging from below detection limits to 10^6 cells/m^2. However, only 0.1 to 55% of these viable cells were able to grow on defined culture medium. Isolated members of the *Bacillaceae* family were more physiologically diverse than those reported in previous studies, including thermophiles (*Geobacillus*), obligate anaerobes (*Paenibacillus*), and halotolerant, alkalophilic species (*Oceanobacillus* and *Exiguobacterium*). Non-spore-forming microbes (α- and β-proteobacteria and actinobacteria) exhibiting tolerance to the selected stresses were also encountered. The multiassay cultivation approach employed herein enhances the current understanding of the physiological diversity of bacteria housed in these clean rooms and leads us to ponder the origin and means of translocation of thermophiles, anaerobes, and halotolerant alkalophiles into these environments.

Clean room environments are of immense value in the fabrication and assembly of many industrial products, including electronics, pharmaceuticals, medical devices, and spacecraft components. The low density of aerosolized particulates within these rooms reduces the amount of both inorganic and biological contamination on and within the assembled products. The reduction of biocontamination and maintenance of a clean environment is of particular importance to the assembly of spacecraft hardware. The search for life on other planets relies heavily on the authenticity of cells and/or biomarkers detected in extraterrestrial samples. Contamination of these samples with organic matter originating on Earth (forward contamination) would inherently confound the interpretation of any such biosignatures discovered. Consequently, current planetary protection protocols require that spacecraft are constructed and assembled under stringent quality-controlled clean room conditions.

Clean room certification is based on the maximum number of particles greater than 0.5 μm per cubic foot of air (ISO 14644-1, Part 1: Classification of air cleanliness; www.iest.org/iso/iso1.htm). The air within class 10 clean rooms is maintained at fewer than 10 particulates per cubic foot, class 10K clean rooms are allowed to harbor a density of 10,000 particles per cubic foot, and so on. In addition to air quality, several environmental parameters of spacecraft-associated clean rooms are also controlled, such as humidity, temperature, and circulation. National Aeronautics and Space Administration (NASA) quality assurance engineers perform periodic audits to ensure that certified-facility cleanliness levels conform to the requirements delineated.

Spacecraft assembly environments are highly selective for microorganisms that tolerate desiccation, chemical oxidizing agents, and UV irradiation (12, 13, 32a, 33), and the remarkably hardy species that persist have the potential to gain access to flight hardware (2, 14, 22, 27, 32). The overall cleanliness of hardware fabricated for missions to Mars, Europa, or Enceladus is of particular concern, as these bodies present the greatest likelihood of sustaining earthly life and affording it the ability to (i) colonize and proliferate and/or (ii) complicate subsequent searches for extraterrestrial life forms (19, 20). In accordance with regulations outlined in international treaties, NASA utilizes a traditional, cultivation-based assay to assess spacecraft cleanliness (18, 20). Although a valid proxy for microbial abundance, this single assay offers only a limited assessment of the phyloge-

* Corresponding author. Mailing address: Biotechnology and Planetary Protection Group, M/S 89, Jet Propulsion Laboratory, California Institute of Technology, 4800 Oak Grove Dr., Pasadena, CA 91109. Phone: (818) 393-1481. Fax: (818) 393-4176. E-mail: kjvenkat@jpl.nasa.gov.

† Present address: Lehrstuhl fuer Mikrobiologie und Archaeenzentrum, Universitaet Regensburg, Universitaetsstrasse 31, 93053 Regensburg, Germany.

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TABLE 1. Locations and characteristics of sampling points

Facility	Location	Dimensions (width × length × ht) (m)	Characteristics[a]	Significance	Sample	Description	Clean room certification[b]	Method of collection[c]	Area sampled	Resuscitated vol (ml)
Johnson Space Center Genesis Curation Laboratory	Houston, TX; Gulf coast; swamp-like	4.55 × 6.3 × 5.55	24.0 ± 3°C; 50 ± 5% RH; ULPA	Genesis Processing and Storage Lab	JSC-1	Lighting fixtures	Class 10	Wipe	0.56 m^2	35
					JSC-2	Subfloor	Class 10	Wipe	0.37 m^2	35
		5.45 × 3.0 × 5.55		Precision Experimental Cleaning Lab	JSC-3	Lighting fixtures	Class 10	Wipe	0.56 m^2	35
					JSC-4	Subfloor	Class 10	Wipe	0.37 m^2	35
					JSC-5	Air duct	Class 1K	Wipe	0.37 m^2	35
		11.4 × 1.8 × 7.0		Corridor connecting JSC-1 through JSC-4	JSC-6	Floor (center)	Class 1K	Wipe	1 m^2	35
					JSC-7	Threshold (inside entrance)	Class 1K	Wipe	0.37 m^2	35
		3.3 × 1.6 × 7.0		Gowning room	JSC-8	Floor of garment changing room	Class 5K	Wipe	0.74 m^2	35
		Not applicable	Not controlled	Receiving area	JSC-9	Floor (outside entrance)	Uncertified	Wipe	0.37 m^2	35
		Not applicable	Not controlled	Emergency entrance	JSC-10	Floor (outside entrance)	Uncertified	Wipe	0.37 m^2	35
Jet Propulsion Laboratory Spacecraft Assembly Facility	Pasadena, CA; West coast; dry desert like	25 × 36 × 15	20 ± 5°C; 40 ± 5% RH; HEPA	Pathfinder (1997); Mars Exploration Rovers (2001)	JPL-1	Floor (center)	Class 100K	Wipe	1 m^2	200
					JPL-2	Floor (inside east entrance)	Class 100K	Wipe	1 m^2	200
Lockheed Martin Aeronautics Multiple Testing Facility	Denver, CO; high altitude; dry	15.85 × 15.85 × 18.29	22.2 ± 3°C; 40 ± 10% RH; HEPA	Mars Reconnaissance Orbiter (2005)	LMA-1	Tread of a stepladder	Class 100K	Swab	25 cm^2	10
					LMA-2	Floor (inside entrance)	Class 100K	Swab	25 cm^2	10
		Not applicable	Not controlled		LMA-3	Tread of a stepladder	Class 100K	Swab	25 cm^2	10
					LMA-4	Floor (outside entrance)	Uncertified	Swab	25 cm^2	10
Kennedy Space Center Payload Hazardous Servicing Facility	Cape Canaveral, FL; East coast; swamp-like	32.61 × 18.39 × 28.91	21.7 ± 3.3°C; 60% max RH; HEPA	Mars Exploration Rovers (2001); Mars Reconnaissance Orbiter (2005)	KSC-1	Floor (northwest corner)	Class 100K	Wipe	1 m^2	35
					KSC-2	Floor (southwest entrance)	Class 100K	Wipe	1 m^2	35
					KSC-3	Floor (center)	Class 100K	Wipe	1 m^2	35
					KSC-4	Floor (north side of bay doors)	Class 100K	Wipe	1 m^2	35
					KSC-5	Floor (south side of bay doors)	Class 100K	Wipe	1 m^2	35
					KSC-6	Top of lockers (southeast)	Class 100K	Wipe	1 m^2	35
					KSC-7	Table top (southeast)	Class 100K	Wipe	1 m^2	35
					KSC-8	Wall (main south entrance)	Class 100K	Wipe	1 m^2	35
					KSC-9	Wall (northwest corner)	Class 100K	Wipe	1 m^2	35
					KSC-10	Floor (inside entrance)	Class 100K	Wipe	1 m^2	35

[a] The temperature, relative humidity (RH), and filter (HEPA, high-efficiency particle filter; ULPA, ultra low particle Air filter) are shown. max, maximum.
[b] Certification is defined by the maximum number of particles of the size >0.5 μm in one cubic foot of air.
[c] Sterile wipes were premoistened with phosphate saline buffer. Sterile swabs were premoistened with phosphate saline buffer. Swaps were used instead of wipes when sample area was small or irregular.

TABLE 2. Methodologies employed to cultivate several extremotolerant bacteria from spacecraft assembly facilities

Type of microbe	Base medium[a]	Alteration to medium and/or additional treatment	Vol of sample (μl)	Plating method	Incubation temp (°C)	Incubation time (days)	Reference
Heterotrophs	R2A		100	Spread	25	7	12
Sporeformers	TSA	Heat shock[b]	5,000	Pour	32	7	18
Acidophiles	R2A	pH 3[c]	100	Spread	25	7	30
Alkaliphiles	R2A	pH 9[d]	100	Spread	25	7	30
Thermophiles	R2A		100	Spread	65	4	This study
Psychrophiles	R2A		100	Spread	4	10	This study
Halophiles	R2A	25% NaCl[e]	100	Spread	25	7	This study
Anaerobes	Thio	Incubated in anaerobic chamber[f]	100	Spread	25	7	This study
UV resistant	R2A	1,000 J/m^2 UV exposure[g]	5,000	Pour	25	7	21
H_2O_2 resistant	R2A	One hour 5% H_2O_2 incubation[h]	834	Pour	25	7	11

[a] Three different base media were used: R2A, BD Difco R2A agar (catalog no. 218263); TSA, BD Difco tryptic soy agar (catalog no. 236950); Thio, BD BBL thioglycolate medium Brewer modified.

[b] Five milliliters of sample was heat shocked for 15 minutes in an 80°C water bath prior to plating.

[c] Four milliliters of a 0.5 M stock solution of citric acid to each 100 ml of R2A agar before pouring the plates. The pH of preautoclaved medium was tested using an Orion Benchtop pH/ISE meter (model 710A).

[d] One milliliter of a 10% stock solution of Na_2CO_3 to each 100 ml of R2A agar before pouring the plates. The pH was confirmed as described in footnote *c*.

[e] NaCl was added to preautoclaved R2A agar until a final concentration of 25% NaCl by mass was achieved.

[f] A BD GasPak anaerobic chamber was used with GasPak anaerobic system envelopes (catalog no. 270304). Anoxity was confirmed with BBL dry anaerobic indicator strips (catalog no. 271051).

[g] Five milliliters of sample were exposed to 1,000 J UV-C light while being stirred with a continuous stir bar in an autoclaved glass petri dish prior to plating.

[h] Sample was incubated with H_2O_2 at a total concentration of 5% H_2O_2 in a sterile glass vial in the dark at room temperature for one hour. Bovine catalase was added (final concentration of 100 μg/ml) to quench the reaction.

netic diversity and physiological breadth of the bacterial population present (12, 13). The utilization of numerous, different cultivation media has been shown to increase observed biological diversity in both artificial and natural environments (6–8). Recently, an investigation involving pharmaceutical production units showed that when culture media were modified to promote the growth of oligotrophs (low-nutrient-loving bacteria), approximately 2 orders of magnitude more bacteria were cultured than with standard minimal media (17). Similarly, increasing the number of medium types employed increased the yield of cultivated ciliate species from a lake in northern England nearly sevenfold (34).

Although culture-independent methods of community assessment, such as 16S rRNA gene-based clone library analyses (15a), have been carried out on samples collected from clean room environments, multiassay cultivation-based examinations have not yet been performed. Such strategies are necessary to ensure the viability of strains detected, and they can yield useful information regarding not only phylogenetic affinities but also physiologic capabilities of the microbes present. The underlying objective of this study was to investigate the occurrence of resident microbiota tolerable of extreme conditions by employing various cultivation methods. This approach, therefore, not only provides a more comprehensive analysis of clean room-associated cultivable bacterial diversity but also demonstrates the relative abundance of physiologically flexible, "hardy" bacteria.

MATERIALS AND METHODS

Sample locations and sampling. Four geographically distinct NASA clean rooms were sampled, the Jet Propulsion Laboratory Spacecraft Assembly Facility (JPL-SAF), the Lockheed Martin Aeronautics Multiple Testing Facility (LMA-MTF), the Johnson Space Center Genesis Curation Laboratory (JSC-GCL), and the Kennedy Space Center Payload Hazardous Servicing Facility (KSC-PHSF). Typical spacecraft assembly facilities consist of both certified clean rooms and associated uncertified locations. The specific sampling locations from each facility and their corresponding class designations are given in Table 1.

All spacecraft assembly facilities examined in this study were maintained with daily cleaning regimens consisting of the replacing of tacky mats, wiping surfaces and support hardware fixtures, and vacuuming and mopping floors using clean room-certified sanitizing agents (disinfectants, alcohol, or ultrapure water). Prior to entering the clean room, staff must take appropriate actions to minimize the influx of particulate matter. Specific entry procedures varied depending on the certification level of the clean room and the presence or absence of mission hardware. Precautions generally included the donning of clean room-certified garments, with cosmetics, fragrances, body spray, and hair gels being prohibited in the clean rooms certified <5K. The air of all facilities was filtered through high-efficiency particle air (HEPA) filters with the exception of JSC clean rooms which utilized ultralow particle air filters to achieve higher cleanliness. In addition, the floors of JSC <5K-certified clean rooms were raised to prevent the accumulation of organic-laden debris.

After the collection of particulates from various surfaces as specified in Table 1, sample-containing wipes and swabs were placed in sterile phosphate saline buffer (Invitrogen, Carlsbad, CA) and were either shaken for 5 min at 200 rpm or vortexed for 2 min at maximum speed (18). Samples were then subdivided for further processing, as shown in Table 2.

Culture-dependent analyses. (i) Cultivation of bacterial population. A variety of cultivation assays were employed as a means of selecting for physiologically diverse microbial populations, as shown in Table 2 or as described in other studies (11, 21, 30). Colonies were counted daily for the duration of the incubation time. Isolates were picked, purified, cultured, and stored at −80°C on Cryobeads (ProLab Diagnostics, Wirral, United Kingdom) for further processing and analysis.

(ii) Screening of bacteria that are tolerant to multiple extreme conditions. Purified isolates arising from one physiological selection assay were exposed to each of the other selective assays (with the exception of UV exposure and H_2O_2 incubation) to test for resilience to multiple stresses. Isolates showing multiple tolerances were selected for phylogenetic identification via 16S rRNA gene sequence analysis (see below).

(iii) Identification of bacterial isolates. DNA was extracted from overnight cultures following either standard lysozyme/organic solvent extraction protocols (9) or via Wizard Genomic DNA purification kits (Promega, Madison, WI). Bacterial small-subunit rRNA genes were PCR amplified using the universal, bacterially biased primers B27F and B1492R, as previously described (12). PCR conditions were as follows: 35 cycles, with 1 cycle consisting of 1 min of denaturation at 95°C, 2 min of annealing at 55°C, and 3 min of extension at 72°C, followed by a 10-min incubation at 72°C. An alignment of ca. 30,000 homologous full and partial sequences available in the public ARB database (15) was used to derive phylogenetic affiliations of the test strains. Novel 16S sequences (ca. 1,400 nucleotides) were aligned to their nearest neighbor using the automated tools of the ARB software suite (Technische Universitat Munchen, Munich, Germany [http://www.mpi-bremen.de/molecol/arb/]). The resulting alignment was checked

TABLE 3. Intracellular ATP content of several microbes that were isolated from spacecraft or other clean environmental surfaces

Organism	Strain[a]	Intracellular ATP content (10^{-18} mol/CFU)	RLU/CFU or RLU/spore	Avg RLU/CFU
Gram-negative bacteria				1.34
Acinetobacter radioresistens	MO-50v1	1.91	0.98	
Escherichia coli	ATCC 25922	2.39	1.23	
Methylobacterium radiotolerans	MSFC 2M5-R1	3.70	1.90	
Proteus mirabilis	ATCC 29906	2.13	1.10	
Pseudomonas aeruginosa	ATCC 27853	1.84	0.95	
Pseudomonas stutzeri	JPL-1	1.44	0.74	
Pseudomonas graminis	JPL-8	5.45	2.80	
Sphingomonas yanoikuyae	MSFC 4M9-R1	1.98	1.02	
Gram-positive bacteria				4.49
Bacillus subtilis	ATCC 9372	8.45	4.34	
Bacillus macroides	JPL-4	10.27	5.28	
Kokuria rhizophila	JPL-9	11.99	6.16	
Micrococcus luteus	IFO 3333	8.28	4.26	
Staphylococcus aureus	ATCC 25923	5.77	2.97	
Staphylococcus capitis	JPL-2	8.85	4.55	
Staphylococcus epidermidis	JPL-7	7.47	3.84	
Yeasts				263.50
Candida utilis	NISL 3727	677.00	348.07	
Saccharomyces cerevisiae	NISL 3398	348.00	178.92	
Spores				0.0016
Bacillus circulans	ATCC 4513	0.0032	0.0016	
Bacillus subtilis	ATCC 6633	0.0037	0.0019	
Lactobacillus buchneri	ATCC 11577	0.0022	0.0011	
Bacillus subtilis vegetative cells				
Autoclaved	ATCC 6633	No RLU[b]	NG[c]	
UV_{254}-killed	ATCC 6633	No RLU	NG	
H_2O_2-sterilized	ATCC 6633	No RLU	NG	

[a] Abbreviations used in strain designations: MO, Mars Odyssey spacecraft; ATCC, American Type Culture Collection; MSFC, Marshall Space Flight Center; JPL, Jet Propulsion Laboratory; IFO, Institute of Fermentation-Osaka, Japan; NISL, Noda Institute for Scientific Research, Noda, Japan.
[b] When measured for bioluminescence after removing extracellular ATP, no luminescence was recorded.
[c] NG, no growth when grown on TSA or R2A agar.

manually and corrected if necessary. Phylogenetic trees were reconstructed via maximum-parsimony and neighbor-joining methods (15).

Culture-independent analyses. (i) ATP-based bioluminescence assay. Total ATP (total microbes) and intracellular ATP (viable microbes) were measured as described previously (12, 31). The dynamic range of this assay is from 5×10^{-12} M to 10^{-7} M ATP, with one relative luminescence unit (RLU) corresponding to 2×10^{-14} M ATP as determined by linear regression analysis of standard curves with known ATP concentrations (31). To verify intracellular ATP measurements and to ensure that extracellular ATP had been fully degraded, a sterile water sample spiked with purified ATP (Sigma, St. Louis, MO) and inactivated *Bacillus subtilis* (Table 3) vegetative cells were subjected to the ATP-eliminating procedure. No signal above background was obtained.

Quantitatively estimating bioburden from measurements of surface-associated ATP burden necessitates establishing basic assumptions about the type and metabolic state of microbes present. Since the concentration of intracellular ATP is highly dependent upon cell volume and size (10), the distribution of bacterial genera within a given sample significantly affects the average ATP content measured per cell. In order to derive average intracellular ATP content, strains isolated from the surfaces of spacecraft (13), assembly facilities (31), and other sources (2a) representative of several microbial classes were cultured overnight at optimum conditions and were assayed for intracellular ATP at each hour of logarithmic growth. Plate count measurements were performed in parallel to correlate RLU and CFU measurements. On the basis of the ATP content of this collection of strains grown under nutrient-rich conditions, the average ATP content per bacterial cell was estimated to be 2.81 ± 1.01 RLU (Table 3). However, because these clean room environments are largely devoid of nutrients and the majority of microbes present are assumed to be metabolically inactive, the application of this value in deriving clean room-associated microbial burden from ATP measurements would result in a gross underestimation. Furthermore, yeast and fungi contain 100 times more ATP than typical gram-negative bacteria, and as shown in Table 3, spores possess negligible amounts of intracellular ATP. In consideration of these facts, conservatively, 1 RLU is presumed to be approximately equal to 1 CFU (12, 31).

(ii) TaqMan quantitative PCR (Q-PCR). rRNA gene copy numbers were quantified in triplicate using an Applied Biosystems 7700 detection system. Universal bacterial primers targeting the 16S rRNA gene, primers 1369F (5′-CGGTGAATACGTTCYCGG-3′) and 1492R (5′-GGWTACCTTGTTACGACTT-3′), and the fluorescence-labeled probe TM1389F (5′ 6-carboxyfluorescein [FAM]-CTTGTACACACCGCCCGTC-6-carboxytetramethylrhodamine [TAMRA]-3′), were used in this quantitative analysis (29). Each 50-μl reaction consisted of 25 μl of 2× TaqMan universal PCR master mix (Applied Biosystems, Inc., Foster City, CA), 0.8 μM of each oligonucleotide primer, 0.5 μM of oligonucleotide probe, and 1 μl of template DNA. Reaction conditions were as follows: 95°C denaturation for 15 min, followed by 40 cycles, with 1 cycle consisting of denaturation at 95°C for 15 s and a combined annealing and extension at 60°C for 1.5 min.

Controls and lower detection limits of assays employed. Appropriate controls were used whenever necessary. Sterile water samples served as negative controls in all culture-based and molecular assays. Pure ATP (Sigma, St. Louis, MO) was decimally diluted and served as a standard curve for ATP analyses. Purified DNA from *Bacillus pumilus* ATCC 7061 was included in the PCR amplification protocols as a positive control. To prevent false-negative results in PCRs associated with the presence of inhibitory substances, a known amount of DNA was extracted from *B. pumilus* and spiked (1 pg per reaction mixture) as an internal standard when necessary. None of the DNA extracts in this study inhibited the PCR. The lower detection limits were 1 CFU ($= 3.5 \times 10^2$ to 2.6×10^3 CFU/m^2) for the cultivable plate count assay, 10 RLU ($= 3.5 \times 10^3$ to 2.6×10^4 CFU/m^2) for ATP analysis, and 100 copies ($= 3.5 \times 10^4$ to 2.6×10^5 CFU/m^2) for Q-PCR analysis. The range of detection limits varies depending on the area sampled (Table 1).

TABLE 4. Microbial characterization of various spacecraft assembly facilities

Location	Clean room certification	No. of microbes/m^2 that were:					% of population that were:		
		Total (A)[a] (RLU)	Viable (B)[a] (RLU)	Cultivable bacteria (C) (CFU)	Cultivable spores (D) (CFU)	Total bacteria (Q-PCR) (no. of copies)	Viable [(B/A) × 100]	Cultivable [(C/B) × 100]	Spores [(D/C) × 100]
JSC-1	Class 10	1.7×10^5	BDL[b]	BDL	BDL	BDL	–[d]	–	–
JSC-2	Class 10	5.0×10^5	BDL	BDL	BDL	2.8×10^6	–	–	–
JSC-3	Class 10	2.8×10^5	BDL	BDL	BDL	BDL	–	–	–
JSC-4	Class 10	2.1×10^7	BDL	BDL	BDL	2.2×10^6	–	–	–
JSC-5	Class 1K	1.2×10^5	BDL	BDL	9.5×10^1	7.0×10^6	–	–	–
JSC-6	Class 1K	1.3×10^7	7.5×10^4	BDL	BDL	BDL	0.6	VBNC[e]	–
JSC-7	Class 1K	2.3×10^6	7.7×10^4	BDL	BDL	3.6×10^6	3.4	VBNC	–
JSC-8	Class 5K	7.0×10^6	1.1×10^5	4.7×10^3	BDL	2.3×10^7	1.6	4.1	–
JSC-9	Uncertified	6.8×10^6	1.0×10^5	4.7×10^3	2.8×10^2	1.6×10^8	1.5	4.6	6.0
JSC-10	Uncertified	2.3×10^6	5.9×10^4	9.4×10^2	BDL	5.6×10^7	2.6	1.6	–
JPL-1	Class 100K	3.4×10^6	3.1×10^4	4.3×10^3	BDL	BDL	0.9	13.6	–
JPL-2	Class 100K	4.5×10^6	4.8×10^4	BDL	BDL	3.3×10^7	1.1	VBNC	–
LMA-1	Class 100K	1.1×10^9	7.4×10^6	8.6×10^6	BDL	ND[c]	0.7	>100	–
LMA-2	Class 100K	7.5×10^7	2.2×10^6	6.0×10^4	BDL	ND	3.0	2.7	–
LMA-3	Class 100K	8.0×10^8	8.0×10^6	2.5×10^6	BDL	ND	1.0	31.4	–
LMA-4	Uncertified	3.6×10^8	3.9×10^6	6.2×10^6	BDL	ND	1.1	>100	–
KSC-1	Class 100K	1.4×10^7	1.9×10^4	8.8×10^2	3.5×10^1	1.4×10^8	0.1	4.7	4.0
KSC-2	Class 100K	1.4×10^7	1.1×10^5	6.3×10^4	1.8×10^3	3.5×10^7	0.8	55.5	2.9
KSC-3	Class 100K	6.0×10^6	8.3×10^3	3.2×10^3	7.0×10^1	9.3×10^7	0.1	37.9	2.2
KSC-4	Class 100K	1.5×10^7	6.4×10^4	9.3×10^3	3.5×10^2	3.2×10^8	0.4	14.4	3.8
KSC-5	Class 100K	4.8×10^6	3.5×10^4	1.4×10^4	5.3×10^2	1.5×10^8	0.7	40.7	3.7
KSC-6	Class 100K	1.4×10^7	3.4×10^5	2.4×10^4	9.1×10^2	5.3×10^7	2.4	7.0	3.8
KSC-7	Class 100K	5.9×10^6	7.0×10^4	3.7×10^3	BDL	3.8×10^7	1.2	5.3	–
KSC-8	Class 100K	1.1×10^6	5.9×10^3	7.0×10^2	2.3×10^2	1.7×10^6	0.5	11.8	35.0
KSC-9	Class 100K	9.3×10^7	1.6×10^5	1.4×10^3	1.8×10^2	2.5×10^6	0.2	0.9	12.5
KSC-10	Class 100K	7.1×10^6	4.2×10^4	1.4×10^4	2.8×10^2	1.2×10^8	0.6	33.1	2.0

[a] Total microbes were estimated on the basis of the total amount of ATP (A), and viable microbes were measured on the basis of intracellular ATP (B). See Materials and Methods for detailed explanations.
[b] BDL, below detection limit of the assay performed.
[c] ND, not determined.
[d] –, value of either numerator and/or denominator is below the detection limit.
[e] VBNC, viable but noncultivable microbes.

Statistical analysis. Statistical analyses were carried out using SPSS for Windows (SPSS, Inc., Chicago, IL). Gaussian distributions were not assumed, and nonparametric statistical tests were performed for each data set. Comparisons of more than two sample sets were carried out using Kruskal-Wallis tests. Mann-Whitney tests with Bonferroni's adjustment were used to distinguish which sample sets differed statistically and significantly. All statistical tests were carried out at the $\alpha = 0.05$ significance level. In addition, appropriate statistical analyses were performed per the manufacturers' instructions, as well as with the Microsoft Excel software package. For example, an additional measurement was taken from samples when the coefficient of variation exceeded 10% for ATP analyses, and standard deviations were calculated from four individual replicates of each sample. Likewise, software included with the Applied Biosystems 7700 detection system was used to generate standard deviations from three replicates of each Q-PCR-amplified sample. The averages of two measurements were calculated for each sample that underwent plate count analysis.

RESULTS

The microbial populations present about the surfaces of various spacecraft-associated clean rooms, as estimated by culture-dependent and -independent analyses, are depicted in Table 4.

Cultivable heterotrophic bacteria. Overall, the LMA-MTF facility locations gave rise to the greatest number (~10^6 CFU/m^2) of cultivable bacteria (grown in unmodified R2A medium [BD Difco R2A agar; catalog no. 218263]) at 25°C; mesophilic heterotrophs), while cultivable bacterial counts ranged from below detectable limits (BDL) to 10^3 CFU/m^2 for the JPL-SAF and from 10^2 to 10^4 for KSC-PHSF surfaces. When JSC samples were categorized according to originating clean room class (class 10, 1K, and 5K), all of the samples collected from class 10 (JSC-1 to JSC-4) and those from 1K (JSC-5 to JSC-7) clean rooms were devoid of any cultivable bacteria. This was not the case, however, with regard to both certified class 5K rooms (JSC-8) and uncertified, exterior entrance floors (JSC-9 and JSC-10), each of which housed 10^3 CFU/m^2. The ratio of cultivable to viable microbes, as described in Table 4, was negligible for samples collected from the class 10 and class 1K clean rooms of JSC, suggesting that these surfaces housed exclusively cells that are viable but not yet cultivable (VBNC).

Figure 1 depicts a phylogenetic tree of all cultivable heterotrophs isolated in this study and identified via 16S rRNA gene sequencing. In total, 51 isolates were recovered spanning 29 known bacterial species and 6 bacterial species yet to be described. These mesophilic heterotrophs clustered into two proteobacterial (α- and γ-proteobacteria) and three gram-positive bacterial classes (actinobacteria, non-spore-forming cocci, and spore-forming rods). Members of the proteobacterial group, representing 9 species, accounted for 25% of the cultivable

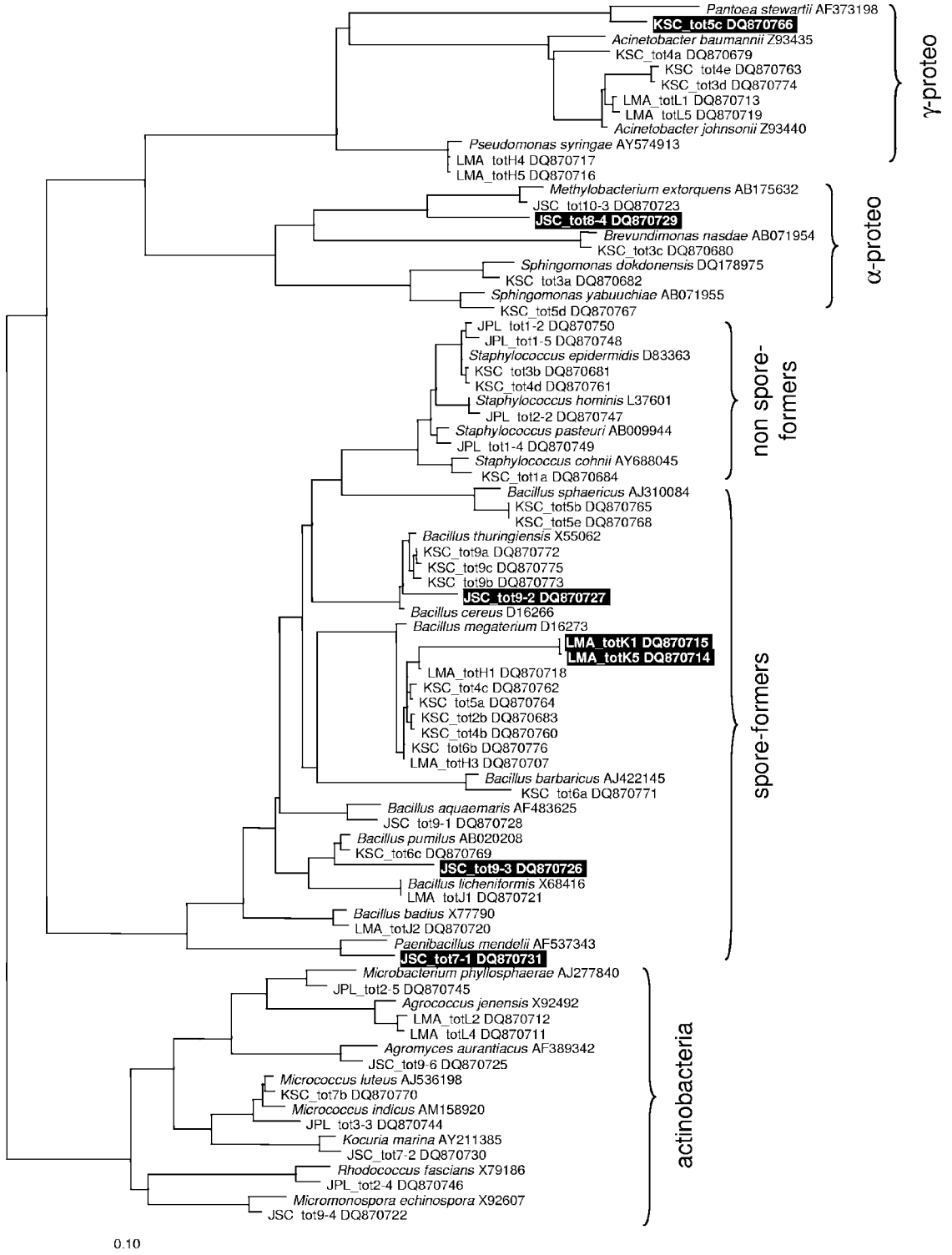

FIG. 1. Phylogenetic tree (maximum parsimony) based on 16S rRNA gene sequences of cultivable bacteria isolated from various spacecraft assembly facilities. The strain designation or type strain of the closest neighbor followed by the GenBank accession number is given. Novel strains identified within this study, showing less than 97.5% similarities (16S rRNA gene) to the closest described species, are indicated by white type on a black background. γ-proteo, γ-proteobacteria; α-proteo, α-proteobacteria. The scale bar shows a 10% estimated difference in nucleotide sequence positions.

bacteria. In contrast, 75% of the cultivable population was shown to belong to gram-positive classes, representing 26 species. Approximately 40% of the cultivable population was identified as members of the genus *Bacillus*, spanning nine distinct species. The most common *Bacillus* species was *Bacillus megaterium*. The JPL locations tested did not bear any spore-forming bacteria; however, non-spore-forming species belonging to actinobacteria and *Staphylococcus* were isolated. Among the samples collected from LMA locations, certified areas consisted of both proteobacterial and gram-positive bacterial groups, whereas uncertified LMA locations housed only *Bacillus* species. Only three isolates from certified JSC locations and six isolates from uncertified locations were retrieved. Two of these isolates were of α-proteobacterial lineage, and all others were identified as gram-positive bacteria. Four of these nine JSC isolates did not belong to any previously described bacterial species and warrant description as novel species. Of the 10 locations sampled at KSC, two did not yield any cultivable bacteria, and 24 strains were isolated and identified from the other eight locations. All five classes of bacteria were represented in these samples, including oligotrophic species of *Sphingomonas* and *Acinetobacter*.

It is generally accepted that if the 16S rRNA gene sequence of an unknown strain is less than 97.5% similar to that of the type strain of its nearest evolutionary neighbor, then the unknown strain represents a novel species (28). There were seven novel mesophilic isolates cultured in this study, representing six novel bacterial species. Two of these novel strains, most closely related to *B. megaterium*, were isolated from the LMA-3 sample, while the KSC-5 location gave rise to a novel species of *Pantoea* (KSC_tot5c). Four of these novel species were retrieved from within JSC facilities, two species from JSC-9 were most closely related to *B. pumilus* (JSC_tot9-3) and *Bacillus cereus* (JSC_tot9-2). Strain JSC_tot7-1 was identified as a *Paenibacillus* sp., and a novel *Methylobacterium* sp. (JSC_tot8-4) was isolated from JSC-8.

Distribution of spore-forming bacteria. In general, spore-forming bacteria were far more scarcely isolated than was expected. There were no spore-forming bacteria cultivated from the LMA-MTF locations despite the fact that these facilities were heavily laden with mesophilic cultivable bacteria. The failure to cultivate sporeformers was also encountered with all of the JPL locations (class 100K) and the class 10 and 5K JSC clean rooms. Of the three samples collected from the class 1K JSC-5 location, only one gave rise to any sporeformers. Even though JSC-9 and JSC-10 locations were not certified, there were no spore-forming bacteria recovered from JSC-10, and sporeformers constituted a mere 6% of the cultivable heterotrophs within JSC-9. KSC samples proved anomalous, as 9 of the 10 KSC-PHSF facility locations presented a spore presence ranging from 3.5×10^1 to 9.1×10^2 CFU/m^2. With the exception of the southernmost facility floor (KSC-2; 1.8×10^3 CFU/m^2), KSC floors (KSC-1 to KSC-5; KSC-10) sustained a relatively low spore burden (2 to 4% of the cultivable heterotrophic population). Much larger proportions of spore-forming bacteria (12.5 to 35%) were recovered from KSC facility walls (KSC-9 and KSC-8). Despite the seemingly ubiquitous distribution of spores within this KSC facility, the surface of a table where spacecraft hardware underwent assembly was devoid of any spore-forming bacteria.

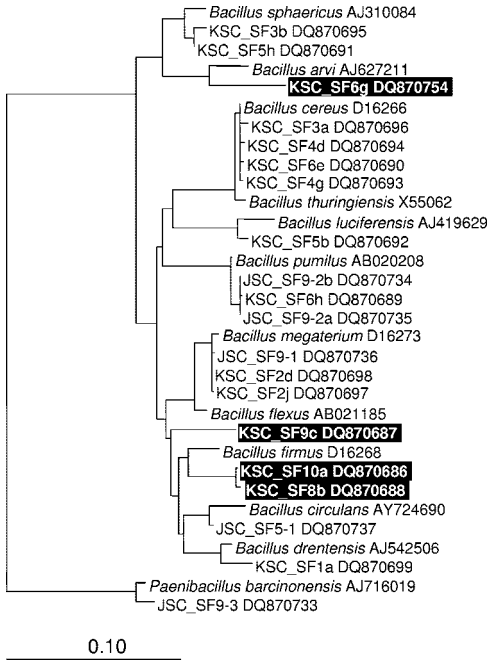

FIG. 2. Neighbor-joining tree showing the phylogenetic positions based on 16S rRNA gene sequences of spore-forming bacteria isolated from various spacecraft assembly facilities. The tree is set up as described in the legend to Fig. 1.

The 16S rRNA gene sequence-derived phylogenetic affiliations of spore-forming bacteria isolated in this study are shown in Fig. 2. There were a total of 20 spore-forming isolates analyzed in this study. Sixteen of these isolates spanned eight known species of *Bacillus*, and an additional four isolates clustered most closely with three novel *Bacillus* species. These novel *Bacillus* strains originated from KSC locations and were most closely related to *Bacillus arvi* (KSC_SF6g), *Bacillus firmus* (KSC_SF8b and KSC_SF10a), and *Bacillus flexus* (KSC_SF9a). There was a lone *Paenibacillus* species encountered in the JSC-9 sample.

Distribution of extremotolerant bacteria. Table 5 summarizes the mean number of clean room isolates capable of tolerating the extreme conditions imposed in this study. Samples collected from LMA and KSC floors, as well as the uncertified JSC locations yielded a wide variety of cultivable bacteria able to tolerate inhospitable conditions. Upon subjecting samples to psychrophilic (growth at 4°C) or halophilic (growth in the presence of 25% NaCl) conditions, no colonies were seen in the media employed. However, alkalotolerant microbes (growth at pH 9.0) were isolated invariably from all locations sampled. LMA-MTF samples exhibited the greatest abundance of alkalotolerant bacteria. In these samples alkalotolerant bacteria accounted for 30% (three certified locations) to 80% (one uncertified location) of all cultivable bacteria. Samples from other locations housed alkalotolerant bacterial populations ranging from 1.6×10^2 to 9.4×10^3 CFU/m^2. Thermophilic bacteria, those bacteria that proliferated 65°C but not at room temperature, were isolated from JPL-2, JSC-9, and KSC-6 locations. LMA-MTF and KSC-PHSF samples yielded bacterial strains capable of growth at pH 3.0; however, these isolates

TABLE 5. Bacterial population of several spacecraft assembly facilities[a]

Type of bacteria[b]	Avg no. (CFU/m^2) of bacterial population from the following spacecraft assembly facilities that were:						
	JSC-GCL				JPL-SAF, class 100K (n = 2)	LMA-MTF		KSC-PHSF, class 100K (n = 10)
	Class 10 (n = 4)	Class 1K (n = 3)	Class 5K (n = 1)	Uncertified (n = 2)		Class 100K (n = 3)	Uncertified (n = 1)	
Thermophiles				2.4×10^2	4.0×10^2			3.5×10^1
Acidophiles						6.7×10^3		5.8×10^2
Alkalophiles	2.4×10^2	1.6×10^2	2.4×10^3	2.1×10^3	6.0×10^2	2.0×10^6	1.2×10^6	9.4×10^3
UV resistant				9.4		2.7×10^3		4.2
H_2O_2 resistant				5.5×10^1				
Anaerobes						6.7×10^3		3.4×10^3

[a] No growth was observed in the psychrophile or halophile selection assay at any location.
[b] The bacteria isolated may be extremotolerant or extremophilic to the condition employed.

did not survive the storage conditions employed in this study. UV-C-resistant bacteria were cultivated from one LMA sample (LMA-1), one JSC sample (JSC-9), and two samples collected from KSC facilities (KSC-5 and KSC-6). There were no UV-resistant strains isolated from JPL locations following exposure of samples to UV-C (1,000 J/m^2). Bacterial isolates tolerating 5% H_2O_2 were recovered from an uncertified JSC partition (JSC-10). Finally, obligate anaerobes were cultivated from LMA-1 and KSC-7 floor samples.

The 16S rRNA gene-derived species identification of bacterial isolates that demonstrated tolerance to one or more of the extreme conditions put forth in this study is shown in Table 6. Of the 17 alkalophiles isolated, 13 (~76%) were gram-positive species, 7 were strains of actinobacteria, 5 were strains of

TABLE 6. Phylogenetic characterization of bacteria that are tolerant to several extreme conditions

Conditions[a]	Location	Certification	Bacterial species	Strain	Accession no.
Alkalophilic (pH 10.6)	JPL-1	Class 100K	*Oceanobacillus* sp.	JPL_AkK1	DQ870753
	LMA-3	Class 100K	*Microbacterium schleiferi*	LMA_AkK1	DQ870710
	LMA-4	Uncertified	*Brachybacterium paraconglomeratum*	LMA_AkL5	DQ870709
	JSC-7	Class 1K	*Bacillus* sp.	JSC_Ak7-1	DQ870739
	JSC-7	Class 1K	*Microbacterium aurum*	JSC_Ak7-2	DQ870743
	JSC-7	Class 1K	*Sphingomonas oligophenolica*	JSC_Ak7-4	DQ870741
	JSC-7	Class 1K	*Sphingomonas trueperi*	JSC_Ak7-3	DQ870742
	JSC-8	Class 5K	*Staphylococcus epidermidis*	JSC_Ak8-2	DQ870740
	JSC-9	Uncertified	*Bacillus pumilus*	JSC_Ak9-3	
	JSC-10	Uncertified	*Bacillus pumilus*	JSC_Ak10-3	
	KSC-2	Class 100K	*Arthrobacter* sp.	KSC_Ak2i	DQ870702
	KSC-2	Class 100K	*Microbacterium arborescens*	KSC_Ak2e	DQ870704
	KSC-2	Class 100K	*Exiguobacterium acetylicum*	KSC_Ak2f	DQ870703
	KSC-3	Class 100K	*Brevundimonas diminuta*	KSC_Ak3a	EF191247
	KSC-4	Class 100K	*Micrococcus mucilaginosus*	KSC_Ak4f	DQ870701
	KSC-6	Class 100K	*Kocuria rosea*	KSC_Ak6a	DQ870700
	KSC-10	Class 100K	*Pseudomonas stutzeri*	KSC_Ak10c	DQ870705
Anaerobic	LMA-1	Class 100K	*Paenibacillus wynnii*	LMA_An	DQ870708
	KSC-7	Class 100K	*Staphylococcus epidermidis*	KSC_Anb	DQ870706
Hydrogen peroxide (liquid 5%)	JSC-10	Uncertified	*Bacillus pumilus*	JSC_Hp10-1	DQ870738
Thermophilic (65°C)	JPL-2	Class 100K	*Geobacillus stearothermophilus*	JPL_T2a	DQ870752
	JPL-2	Class 100K	*Geobacillus kaustophilus*	JPL_T2d	DQ870751
	JSC-9	Uncertified	*Geobacillus thermodenitrificans*	JSC_T9a	DQ870732
	KSC-6	Class 100K	*Bacillus coagulans*	KSC_T6a	EF191245
	KSC-6	Class 100K	*Geobacillus caldoxylosilyticus*	KSC_T6b	DQ870685
UV-C (1,000 J m^{-2})	LMA-1	Class 100K	*Bacillus simplex*	LMA_UVH-1	EF191246
	JSC-9	Uncertified	*Bacillus megaterium*	JSC_UV9-1	DQ870724
	KSC-5	Class 100K	*Bacillus cereus*	KSC_UV5a	DQ870755
	KSC-5	Class 100K	*Bacillus cereus*	KSC_UV5b	DQ870756
	KSC-5	Class 100K	*Nocardioides* sp.	KSC_UV5c	DQ870757
	KSC-6	Class 100K	*Bacillus pumilus*	KSC_UV6i	DQ870758
	KSC-6	Class 100K	*Paenibacillus illinoisensis*	KSC_UV6c	DQ870759

[a] The bacteria isolated may be extremotolerant or extremophilic to the condition employed.

TABLE 7. Characterization of strains tolerant to multiple extremes isolated from various spacecraft facilities

Bacterial species	Strain	Source	Clean room certification	Nearest neighbor (16S rRNA gene sequence similarity [%])	Original isolation method as detailed in Table 2	Growth of bacteria[a] under the following conditions:															
						Temp (°C)				MgCl (g/100 ml)			NaCl (g/100 ml)					pH				
						25	32	50	65	10	20	35	5	10	15	20	25	3.0	5.1	7.4	9.1	10.6
Geobacillus thermodenitrificans	JSC_T9a	JSC-9	Uncertified	*G. thermodenitrificans* (99)	Thermophiles	−	−	+	+	+	+	−	−	−	−	−	−	−	−	+	+	−
Geobacillus thermodenitrificans	Type strain	ATCC[b]				−	−	+	+	+	−	−	−	−	−	−	−	−	−	+	−	−
Oceanobacillus sp.	JPL_Ak1	JPL-1	Class 100K	*O. iheyensis* (97)	Alkalophiles	+	+	−	−	+	+	+	+	+	+	+	−	−	−	+	+	+
Oceanobacillus iheyensis	Type strain	ATCC				+	±	−	−	+	+	+	+	+	+	+	−	−	−	+	+	−
Exiguobacterium acetylicum	KSC_Ak2F	KSC-2	Class 100K	*E. acetylicum* (99)	Alkalophiles	+	+	−	−	+	+	+	+	+	−	−	−	−	+	+	+	+
Exiguobacterium acetylicum	Type strain	ATCC				+	+	−	−	+	+	+	+	+	−	−	−	−	−	+	+	+
Bacillus sp.	KSC_SF10a	KSC-10	Class 100K	*B. firmus* (96)	Sporeformers	+	+	−	−	+	+	+	+	−	−	−	−	−	−	+	+	+
Bacillus sp.	KSC_SF8b	KSC-8	Class 100K	*B. firmus* (96)	Sporeformers	+	+	−	−	−	−	−	+	+	+	−	−	−	−	+	+	+

[a] Symbols: +, growth; ±, weak growth; −, no growth.
[b] ATCC, American Type Culture Collection.

spore-forming rods, and one strain was *Staphylococcus epidermidis*. The other four alkalophilic strains were identified as members of the α-proteobacteria (three strains; *Brevundimonas* and *Sphingomonas*) and γ-proteobacteria (*Pseudomonas stutzeri*). Alkalotolerant, actinobacterial isolates belonged to the genera *Arthrobacter*, *Brachybacterium*, *Kocuria*, *Microbacterium*, and *Micrococcus*. Alkalotolerant spore-forming rods were species of *Bacillus*, *Oceanobacillus*, and *Exiguobacterium*. Three alkalotolerant isolates represented novel bacterial species, based on 16S rRNA gene sequence dissimilarity, and were most closely related to species of *Arthrobacter* (KSC_Ak2i), *Bacillus* (JSC_Ak7-1), and *Oceanobacillus* (JPL_Ak1). In total, 14 alkalotolerant strains were isolated from JSC (7 strains) and KSC (7 strains) locations. In contrast, only one strain was isolated from JPL and two strains from LMA facilities. None of the locations of JSC class 10 clean room samples contained alkalotolerant bacteria. Two obligately anaerobic strains isolated in this study were identified as *Paenibacillus wynnii* (LMA_An) and *Staphylococcus* sp. (KSC_AnB). The one isolate demonstrating tolerance to 5% liquid H_2O_2 was identified as *B. pumilus* (JSC_Hp101). Species of *Geobacillus* accounted for four of the five thermophilic strains isolated; the other one strain was identified as *Bacillus coagulans*. These *Geobacillus* isolates could not be identified at the species level, since most *Geobacillus* species are too closely related to resolve. Like the thermophilic isolates, all UV-C resistant strains were spore-forming rods of the genera *Bacillus*, *Paenibacillus*, and *Nocardioides* (KSC_UV5c, a novel actinomycete).

Characterization of bacterial strains that tolerate extreme conditions. A total of 32 isolates showed significant growth in at least one of the above-described extreme culturing conditions, and each of these isolates was further analyzed for thermotolerance (>65°C), halotolerance (25% NaCl, 20% $MgCl_2$), acidotolerance (pH 3.0), and alkalotolerance (pH 9 and 10.6). Table 7 summarizes the survival capabilities of the five isolates that exhibited the broadest ranges of tolerance. In addition to the wild-type strains isolated over the course of this study, corresponding type strains were procured from culture collections and were subjected to identical challenges for comparison. Both the thermophilic strain *Geobacillus thermodenitrificans* JSC_T9a and its type strain, *G. thermodenitrificans* DSM 465^T, showed prolific growth at 65°C but failed to grow at temperatures of <50°C. However, the wild-type *G. thermodenitrificans* JSC_T9a strain grew both in the presence of 20% $MgCl_2$ and at pH 9, while type strain DSM 465^T did not. The novel, alkalophilic *Oceanobacillus* sp. (JPL_Ak1) propagated well at pH 10.6, whereas the *Oceanobacillus iheyensis* type strain ATCC BA000028^T, isolated from a depth of 1,500 m below the sea surface, failed to grow at this elevated pH. Both strains grew in liquid media augmented with either 20% NaCl or 35% $MgCl_2$. *Exiguobacterium acetylicum* KSC_Ak2F and its type strain counterpart NCIB 9889^T exhibited significant growth at both pH 10.6 and in the presence of 35% $MgCl_2$, but neither of these strains could grow in the presence of >10% NaCl. Two novel spore-forming isolates cultured following heat shock (Table 2) exhibited growth at pH 10.6. Strain KSC_SF10a grew in the presence of 35% $MgCl_2$, but the KSC_SF8b strain did not. The 1.5-kb 16S rRNA gene sequences of these two novel *Bacillus* strains, isolated from different KSC locations (one from the wall, KSC-8; the other from floor, KSC-10; Table 1) were identical and are most closely related to *B. firmus* (96% 16S rRNA gene sequence similarity). All of the strains that demonstrated tolerance to multiple extreme conditions were spore-forming bacteria.

Total microbial population. The total microbial burden, as derived from measurements of total ATP, was 10^6 for JPL, 10^8 to 10^9 for LMA, 10^5 to 10^7 for JSC, and 10^6 to 10^7 RLU/m^2 for KSC locations. LMA-MTF locations contained the highest total ATP content, implying the greatest amount of organic matter (though not necessarily viable cells) among all of the class 100K clean room samples. No relationship was observed between certified LMA-MTF (LMA-1 to LMA-3) and uncertified (LMA-4) locations and the number of total microbes. However, the JSC locations that were maintained at class 10 to 5K showed the least number of total microbes (10^5 RLU/m^2 for four out of eight locations) of all locations tested. When Q-PCR targeting the 16S rRNA gene was used to assess total bacterial contamination, no statistically significant correlation was found between certification and bacterial burden (Table 8). The bacterial contamination (both dead and alive), as mea-

TABLE 8. Statistical analysis to compare the microbial populations of various certified clean rooms

Biological assay	*P* value						
	Class 100K and <100K[a]	JPL and JSC[b]	JPL and LMA[b]	JPL and KSC[b]	LMA and JSC[b]	LMA and KSC[b]	KSC and JSC[b]
Total microbes (total ATP)	0.017*	0.602	0.064	0.083	0.062	0.007*	0.062
Viable microbes (intracellular ATP)	0.025*	0.577	0.064	0.667	0.097	0.005*	0.097
Cultivable heterotrophic bacteria	0.001*	0.351	0.064	0.282	0.001*	0.002*	0.001*
Cultivable spores	0.023*	0.351	0.157	0.067	0.002*	0.480	0.002*
Total bacteria (Q-PCR)	0.160	0.686	0.157	0.086	0.006*	0.058	0.006*

[a] *P* values are for differences in each assay between certified spacecraft assembly facilities. Significance level, $\alpha = 0.05$. Significant results are indicated (asterisks).
[b] *P* values are for differences between specific spacecraft assembly facilities. Significance level, $\alpha = 0.0083$ after Bonferroni adjustment. Significant results are indicated (asterisks).

sured by the Q-PCR assay, was BDL to 10^7 for JPL, BDL to 10^8 for JSC, and 10^6 to 10^8 copies/m^2 for KSC locations. In addition, four locations did not yield any 16S rRNA gene copy numbers (three JSC samples and one JPL sample), and all DNA extraction attempts failed for the samples collected from the LMA-MTF locations. Due to this inconsistency, comparisons of 16S rRNA gene copy numbers with corresponding CFU or ATP values were not attempted.

Viable microbial population. For the JSC facilities, the intracellular ATP content (indicative of viable microbes) of clean room floors correlated well with the certification level of the clean rooms examined (Table 8). None of the JSC class 10 samples yielded viable microbes, as measured by intracellular ATP analysis. One of the three samples collected from JSC class 1K clean rooms did not yield any viable microbes, while samples from the other two class 1K clean rooms (JSC-6 and JSC-7) exhibited a viable microbial burden of 10^4 RLU/m^2. Samples collected from the sole class 5K clean room and samples collected from the uncertified JSC locations exhibited $\sim 10^5$ RLU/m^2. Among the other facilities, all of which are class 100K clean rooms, JPL samples yielded 10^4 RLU/m^2, followed by KSC (10^3 to 10^5 RLU/m^2) and LMA (10^6 RLU/m^2). For the LMA samples, no discernible pattern in microbial abundance (Table 4) was evident between the certified (LMA-1 to LMA-3) and uncertified rooms (LMA-4). In addition, among the various types of samples collected from the KSC-PHSF (floor, table, top of the lockers, and wall), no apparent difference in viable microbial numbers was observed (Table 4).

Statistical evaluation of bacterial abundance. Enumeration data for the four spacecraft assembly facilities described in this study were analyzed both with respect to clean room certification and in comparison across assembly facilities. When comparing the 100K class clean rooms (KSC, LMA, and JPL) to those certified below 100K (JSC), significant differences were found between the two clean room categories with respect to median values of intracellular ATP ($P = 0.025$), total ATP ($P = 0.017$), cultivable mesophilic heterotrophs ($P = 0.001$), and cultivable spores ($P = 0.023$). No significant difference in median Q-PCR values was detected between the two assembly facility categories ($P = 0.160$). Examination of the same data with respect to geographic location confirmed that differences in bioburden are generally observed between clean rooms such as JSC and class 100K rooms with lower particle burdens ($P < 0.0083$). All comparisons of 100K certified facilities detected no significant differences in microbial abundance, with the exception of measurements of intracellular ATP, total ATP, and cultivable bacteria taken from the LMA and KSC 100K facilities ($P = 0.005$, 0.007, and 0.002, respectively).

DISCUSSION

Historically, the vast majority of attempts made at enumerating microbes present on clean room surfaces have been carried out using traditional, culture-based techniques (1, 4, 5, 17, 23–26, 31, 32a). This has been largely due to the lack of rapid and inexpensive culture-independent methods, in addition to the difficulty involved with differentiating dead and live microbes. Microscopy, for example, is not practical for use in assessing these surfaces because of the drastically low number of microorganisms (0 to 10^2 CFU per 25 cm^2) present (13). Moreover, confounding factors, such as autofluorescence, false-positive results, nonspecific binding of stain, and false-negative results resulting from insufficient penetration of stain (16) undermine the value of microscopy in evaluating the microbial burden of even relatively highly laden clean room floor samples, which house cell densities 2 orders of magnitude greater than spacecraft surfaces (13). While cultivation circumvents many of these issues and offers a straightforward means of enumerating some portion of viable microbial population via colony counting, its usefulness is inherently limited, as only a minor fraction of all known microorganisms is detectable with any single medium. This study employed 10 different media as a means of broadening the overall detection spectrum afforded by this propagation-based method.

Recently, a rapid intracellular ATP assay has been developed that estimates the number of VBNC organisms present on a surface, based on approximations of average cellular ATP content and measured ATP concentrations per surface area (12, 31). The ATP assay has long been recognized as being "by far the most convenient and reliable method for estimating total microbial biomass in most environmental samples" (10). In many of the samples originating from clean room floors, the concentrations of unbound, extracellular ATP were as much as 2 to 3 orders of magnitude greater than the concentrations of intracellular ATP. The source of this ATP is most likely degraded human skin cells and other such biomatter. The present study validates the use of ATP as a proxy for cellular viability when exogenous ATP can be excluded from the analysis. While the authors are not claiming that the ATP assay will eliminate and/or circumvent all of the biases associated with assessing

microbial abundance, this technique does in fact provide a rapid estimation of both total and viable microbes present.

The analysis of ratios between intracellular ATP content, total ATP, and CFU counts has proven invaluable in assessing relative microbial abundances about the spacecraft and associated surfaces (12, 31). The ratios of intracellular ATP content to total ATP content were very low (0.2% to 3.4%) in all samples irrespective of clean room certification. Ratios of CFU to viable microbes were also very low and were observed to correlate well with the certification level of the JSC clean rooms (Table 4). The ultralow particle air filter system employed to keep JSC facilities operating within their class 5K certification may also be contributing to the ultraclean condition of this facility by limiting the number of cells entering the facility on airborne particulates. The CFU/intracellular ATP ratio was much higher for KSC floors (median value of 35.5% for six locations [KSC-1 to -5 and KSC-10]) compared to the walls, lockers, and table tops of the same facility. This difference is likely attributable to the accumulation of dust particles on the floors. Anomalously, less than 10% (2 of 26 samples) presented cultivable counts in excess of that which was predicted by analyzing the viable microbial population via intracellular ATP content (Table 4). Such outliers demonstrate that the distribution of microbes in any given sampling location is far from uniform, which renders drawing statistical conclusions challenging (31).

A statistically significant association was found between the levels of clean room certification and the abundance of mesophilic heterotrophs (Table 8). As expected, none of the samples collected from the JSC class 10 or 1K clean rooms yielded any CFU. A similar trend in the absence of viable microbes was observed for all samples originating from class 10 facilities, whereas VBNC bacteria were prominent in the two of the three samples collected from class 1K clean rooms. One of these VBNC-rich samples (JSC-7) was subjected to molecular microbial diversity analysis via direct DNA extraction and subsequent PCR and 16S rRNA gene cloning and sequencing (15a) in an attempt to document the presence of previously reported VBNC bacteria. Most of the resulting bacterial clones belonged to (i) any of several bacterial species requiring different growth media than was employed in this study (*Corynebacterium aurimucosum*; (35), (ii) bacterial species known to be oligotrophic (*Sphingomonas* sp.), (iii) species of symbiotic bacteria (*Veillonella parvula*), or (iv) bacterial species yet to be cultured (*Aerosphaera taetra*). Heterotrophic bacteria were 2 to 4 log units more abundant in the LMA-MTF facility samples ($\sim 10^6$ CFU/m^3) than any other facilities assessed. As mentioned above, particulates may very well be the vehicle for microbial migration into clean rooms, but other sources such as human activity and routine maintenance procedures cannot be ruled out. Sufficient information is not available to draw clear conclusions as to why the LMA-MTF samples yielded such an elevated bacterial burden; however, the presence of appreciable levels of organic matter (2 to 3 log units more total ATP than intracellular ATP [Table 4]) could be promoting the proliferation of cultivable bacteria in this environment.

Previous studies have reported species of the extremely hardy, spore-forming *Bacillus* genus to be the most highly represented (>85% of the cultivable population) in samples collected from spacecraft assembly facilities (13, 23). However, these findings are heavily influenced by measures that optimize the detection of spores (and exclude vegetative cells), such as heat shock procedures and the pour plating of large sample volumes. The findings of this study indicate a far lower preponderance of spore-forming bacteria in these environments (Table 4). Despite being heavily laden with cultivable bacteria, samples collected from LMA-MTF clean rooms were devoid of sporeformers. This could be a result of differences in sample collection, as only a small area (25 cm^2) of the LMA-MTF clean room was sampled compared to other facility locations (0.37 m^2 to 1 m^2). With the exception of one of the three samples collected from the JSC class 1K clean room (Table 4), no spores were retrieved from any of the JPL (class 100K) or JSC (class 10 to 5K) clean rooms. The absence of spore-forming bacteria on KSC table surfaces where spacecraft hardware is assembled suggests that appropriate cleaning and maintenance are in place for the work areas of greatest pertinence to planetary protection.

This is the first study to demonstrate that not only spore-forming *Bacillus* species but also a diverse suite of equally "hardy," physiologically flexible bacterial species persist in the inhospitable conditions of clean room environments. The significant differences observed in the cultivable bacterial populations among the certified clean rooms are more likely attributable to the amounts of human activity and/or routine maintenance of each facility, rather than geographic location. The isolation of numerous bacterial species (including novel organisms) capable of surviving diverse, unfavorable environmental conditions is a testament to the remarkable distribution of physiologically diverse unicellular life. The occurrence of thermophiles (*Geobacillus* spp.) in mesophilic condition, obligate anaerobes (*Paenibacillus* sp.) in oxygen-rich environments, and halotolerant alkalophiles (*Oceanobacillus* sp. and *Exiguobacterium* sp.) in neutral pH environments supports the notion that "everything is everywhere, the environment selects" (3).

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